Polysaccharide (Xanthan) of *Xanthomonas campestris* NRRL B-1459: Procedures for Culture Maintenance and Polysaccharide Production, Purification, and Analysis

ARS-NC-51
November 1976

AGRICULTURAL RESEARCH SERVICE • U.S. DEPARTMENT OF AGRICULTURE

Abstract

Procedures developed and used at the Northern Regional Research Center are detailed for all aspects of producing and analyzing the extracellular anionic heteropolysaccharide of *Xanthomonas campestris* NRRL B-1459. Special precautions in use of the micro-organism, which is a plant pathogen, are indicated. Compositions of media are given for culture maintenance, inoculum buildup, and production in batch cultures; and conditions are described for conducting these operations. Culture maintenance by nonpropagative or semipropagative procedures is advised to insure consistent production of high quality xanthan in good yield.

Procedures and equipment are considered for three scales of operation—laboratory, semi-pilot plant, and pilot plant—for batch fermentation as well as for isolation and purification of xanthan. Methods are given for analyzing culture fluids, for evaluating quality of the xanthan product, and for determining constituent sugars and content of *O*-acetyl and pyruvate substituents. The basic principles and much of the specific detail stated for *Xanthomonas campestris* NRRL B-1459 apply also to the other microbial strains and their extracellular polysaccharides that have been discovered and developed at NRRC.

Keywords: Polysaccharide B-1459, Xanthan, Polysaccharide (xanthan) of *Xanthomonas campestris* NRRL B-1459: maintenance and xanthan fermentation, Extracellular microbial polysaccharide, xanthan: production and analytical procedures

Contents

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The initial research publications from the Northern Regional Research Center (NRRC) on xanthan and its production came out in 1961. Since then a number of modifications and improvements have been made in procedures for culture maintenance and polysaccharide production and recovery. This information is significant for the industrial organizations in both the United States and Europe that either are producing xanthan or are considering production of xanthan or other microbial polysaccharides based on xanthan as a model. To make this information available, our procedures as now recommended are summarized here; and the original research publications are cited for further detail. Cited also are reviews on the fundamental properties of xanthan and its solutions. These properties must be considered in the design and operation of equipment for fermentation, recovery, and use of xanthan.

The basic principles and much of the specific detail included here, although stated for *Xanthomonas campestris* strain NRRL B-1459, are applicable also to the other microbial strains and their extracellular polysaccharides that have been discovered and developed in research at NRRC.

The Micro-Organism

Plant Pathogenicity and Strain History

Most species of *Xanthomonas* pathogenize specific plant hosts. *Xanthomonas campestris* (Pammell) Dowson causes vascular disease of cabbages, cauliflower, and rutabagas (*2*)[2]. The exact origin of *X. campestris* strain NRRL B-1459 is not known. In February 1953, it was sent to the ARS Culture Collection at NRRC by Dr. W. J. Zaumeyer, of the former Bureau of Plant Industry and Soils, U.S. Department of Agriculture, Beltsville, Md. Dr. Zaumeyer, who described the strain as "strongly pathogenic," had no written record of its origin, but his recollection in January 1959 was that he received it from Professor J. C. Walker, Department of Plant Pathology, University of Wisconsin, Madison.

Strain NRRL B-1459 has been deposited in the American Type Culture Collection as ATCC 13951.

Caution in Use of the Micro-Organism

Effective precautions must be observed at various stages of procedure to prevent dissemination of live cells of this plant pathogen as follows:

Opening lyophil tube

The vacuum in the tube must be dispelled before the tube is broken by touching the molten end of a glass rod to a file scratch made in the center of the tube. Then the tube is wiped with cotton moistened with alcohol, broken, and *lightly* flamed before transfer of the pellet to broth.

Exhausting air from fermentors

To prevent entrained bacteria from escaping to the atmosphere, a carbon or glass wool filter is placed in the air exhaust line.

Inactivating cells at end of fermentation

The procedure chosen depends on the scale of operation and the intended use of the product. The fermented

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[2]Italic numbers in parentheses refer to References, page 13.

broth, at pH 5.5 to 6.0, may be heated to 90° C and cooled rapidly. Dilution of the broth with an alcohol-chloroform mixture (*22*) or with alcohol (*37*) before clarifying by centrifugation or filtration, is effective. A biocide may be added to the fermented broth, or the centrifuged cells may be killed by a biocide or steam (*37*) before discarding. Cationic biocides may be expected to react with xanthan, which is anionic.

The killed cells have no known value. Their weight is small, about 15 g per 100 g xanthan produced (*40*).

Strain Variability, Selection, and Maintenance

Colonial variation is a recognized characteristic in *Xanthomonas* species (*4* and references cited therein). Since inception of research on polysaccharide from this strain at NRRC in 1956, substrains have been isolated from stock cultures in which variant forms had developed. In this publication, the general designation strain NRRL B-1459 is applied to the original and any substrain when more specific identity is considered unimportant.

From 1967 through 1975, the substrain NRRL B-1459A was used for research at NRRC (*32,40,41*) and for lyophilized preparations for distribution. This substrain derived from a single, large colony selected from an old stock culture that had developed small variant colonies (*4*). Subsequent culturing of this large colony resulted almost exclusively in large colonies that had normal mucoid character and were yellow. The satisfactory performance of the substrain was established for polysaccharide production under Fermentation Conditions (see Productivity test, page 4). Further research, however, has shown that selection of a substrain must be based not only on its productivity (as indicated by culture viscosity and polysaccharide yield) but also on the quality of the product (*4*). The critical indicator of polysaccharide quality appears to be the pyruvate content. In xanthan from well-defined strains, pyruvate content appears to be stoichiometrically related to the repeating unit of the macromolecule (*25*).

A substrain for replacement of NRRL B-1459A has been selected on the basis of these criteria and related considerations. The polysaccharide from this substrain has the characteristics reported for the product from the parent strain NRRL B-1459 (*21,22,48*).

Evidences of strain variability (*4,23,38,41*) have led to renewed investigation of the role of maintenance procedures in culture stability and variation. Continued investigation and observation will be necessary for thorough evaluation of maintenance procedures because slow change is a subtle factor in culture regression. Suitable maintenance techniques will permit empirical control of strain variation. Ultimate prevention of the degeneration, however, depends on clarifying the basic cause of the instability, which may have metabolic or genetic origins (*23*).

The conclusion reached thus far is that the propagative maintenance procedures previously employed are not advisable for general use but only for necessary culturing over limited time. This conclusion is based on available data (*4,23,38*), recognized theory (*23*), and a growing conviction among microbial geneticists that preservation of desired physiological characteristics usually cannot be assured if maintenance involves repeated culture transfer (serial subculture).

Lyophilization has been used for many years for long-term preservation of bacteria and other micro-organisms at NRRC (*13*) and at other culture collections (*27*). Lyophilization, however, cannot be assumed to be routinely applicable to all microbial strains (*12,23*). This fact has not been considered systematically in relation to strain NRRL B-1459. Factors that might influence the preservation of viability and special physiological characteristics of an individual strain include pre-drying cultural conditions and the growth-cycle stage of the culture as well as the suspending fluid and conditions for preliminary freezing, drying, and sealing (*12,27*).

Prerequisite for strain NRRL B-1459 that shows variation in colonial size and in polysaccharide composition and properties (*4,23,38,41*) is the assured presence of the desired characteristics in the culture at the time of preservation. If facilities are available for making large numbers of lyophil preparations, these inocula may be used also for all production work.

Drying inoculum-size amounts of culture on paper strips provides a simple, convenient technique for shorter-term nonpropagative maintenance of working stock cultures (*23*). A fresh lot of these paper-strip inocula is made from a lyophil preparation about every 3 months. These inocula can be prepared without special equipment, require small storage space (at about 4° C), and, if desired, can be easily distributed. The limited observations made thus far on strain NRRL B-1459 have been favorable but need verification by further application and long-term observation. Preservation of inocula by drying on paper strips, although not applied previously to strains producing extracellular polysaccharides, is comparable in principle to techniques used for microorganisms requiring special care (*27*). It is similar to the procedure of Sordelli (*27*), which, when applied to 36 *Xanthomonas* strains, resulted in 79 percent of the cultures remaining viable up to 14 years (*35*).

Media for Culture Maintenance, Inoculum Buildup and Xanthan Production

YM Agar and Broth

Yeast malt (YM) agar[3] for culture maintenance is prepared with the following (*13*):

	Grams
Yeast extract	3
Malt extract	3
Peptone	5
Glucose	10
Agar	20
Distilled H_2O	1,000 ml
pH not adjusted	

YM broth is made by omitting agar from this composition.

T2GY Agar

The glucose concentration specified for the composition designated T2GY,[3] which is twice that of the standard composition designated TGY (*13*), is necessary to assure consistent and reproducible polysaccharide production by strain NRRL B-1459 (*4*). This modified composition was first reported in 1970 (*40*) and later in 1972 (*41*).

	Grams
Tryptone	5
Yeast extract	5
Glucose	2
K_2HPO_4	1
Agar	20
Tap H_2O	1,000 ml
pH adjusted to 7.0	

Production Medium

	Grams
Glucose	
Commercial (about 89% pure)	22.5
(D-glucose equivalent)	20.0
Distillers' dried solubles	8.0
K_2HPO_4	5.0
$MgSO_4$	.1
Tap H_2O to make volume to 1,000 ml.	
Adjust pH within range 7.0 to 7.2 with sulfuric acid.	

Almost all NRRC research on xanthan production has used distillers' dried solubles which, in initial research, gave better yields of polysaccharide than other complex nitrogen sources tested (*37*). Urea may partially replace distillers' dried solubles (*31*). A clear production medium containing an extract of distillers' dried solubles and urea also has been developed (*40*). By suitable adjustment of total medium composition and other fermentation conditions, however, other sources of growth factors and nitrogen might be used successfully. Examples are yeast preparations, distillers' slop, extracted soybean meal, and milk whey (*5*).

Media Sterilization

Media in laboratory glassware is autoclaved at 121° C and then cooled rapidly with sterile air. Sterilizing time for test tubes and 300-ml Erlenmeyer flasks is 15 min, and for Fernbach flasks, 20 min.

To produce medium, glucose is sterilized separately at pH 4.0 to 4.5 and combined aseptically with the remainder of the sterile medium.

For large-scale operations, details have been published (*16,30,36,37*).

Culture Maintenance

A lyophil preparation of strain NRRL B-1459 usually is sent in response to requests for a culture. Instructions are enclosed for opening the tube to prevent scattering pathogenic cells and to assure asepsis (see Opening lyophil tube, page 1).

Criteria Prerequisite to Lyophilization of Strain NRRL B-1459

Cultures of strain NRRL B-1459, before preservation by lyophilization at NRRC, have met criteria under the conditions specified, as follows:

Culture purity test

Incubate at 25° to 28° C for 24 h in YM broth or on YM agar or T2GY agar slants; streak several YM agar plates and incubate 72 h at 25° to 28°. All colonies should be large [4-mm diameter after 72-h growth at 28° on YM plates (*23*)], mucoid, and light-yellow. Two variant forms of strain B-1459 (*4,23,40,41*)

[3]Agar slant and plate media should be less than 1-month old when used.

should not be present: an intermediate size [about 2-mm diameter after 72-h growth], mucoid, and darker yellow; and small size [about 1-mm diameter after 72-h growth (*23*)], nonmucoid. Although relative sizes will be apparent, some variation in measured diameter of colonies may be observed depending on temperature of incubation and age of culture that is plated (*4*). Other cells (contaminants) should not be present.

Productivity test

Using a 20 to 24 h culture slant from the culture purity test to initiate buildup of inocula, follow Procedure A, page 5. The resultant culture in the Fernbach flask should develop at least 6,800 cP viscosity in 48 h when measured as stated in Fermentation Conditions, page 7.

Polysaccharide quality test

This test requires an isolated, purified sample of polysaccharide from such a completed fermentation as that for the Productivity Test. Details for isolating a test sample of approximately 100 mg specifically for pyruvic acid analysis are described in Isolation and Purification of Xanthan, page 8. The pyruvic acid analysis is described in Analytical Methods, page 10. For best quality xanthan, the pyruvic acid content should be above 4 percent. Such high-quality properties of the polysaccharide solutions as viscosity, rheology, and response to salt correlate with these pyruvic acid values (*38*).

Procedures for Culture Maintenance

Three procedures are described:

Procedure 1: Lyophilization, nonpropagative preservation

Equipment and details of operation have been described (*13,27*).

The steps involved in making a new set of lyophil tubes are diagrammed in figure 1, and the relation is shown to the two other procedures for maintenance.

Procedure 2: Paper-strip preservation, nonpropagative[4]

Initial steps in this procedure are diagrammed in figures 1 and 2. Preliminary preparations are carried out as follows:

a) From Whatman filter paper 3MM cut 30 strips 4 × 50 mm (or 1/8 × 2 in). Soak overnight at 20° to 21° C in 20 m*M* disodium salt of ethylene diamine tetraacetic acid (EDTA, Na_2), then wash for 2 days in five changes of deionized water. Dry in an oven at 100°. Place the strips in a vial (23 mm × 105 mm) with heat resistant cap and sterilize (121°, 15 min, steam).

b) Clean and dry some Fiberglas (Pyrex Brand Wool) as described for paper strips.

c) In a duplicate vial (as for paper strips), place a layer (about 25-mm or 8 g) of Molecular Sieve Adsorbent[5] (8 to 12 mesh beads, 4 Å pore size) and cover with cleaned glass wool. Other dust-free, zeolite-type molecular sieves might be used. Sterilize at 121° C for 15 min, oven-dry at 100°, and store over $CaSO_4$ dessicant until needed.

Suspend contents of one tube of lyo culture in 7 ml sterile YM broth and incubate 16 h or to good growth at 28° C as described in Procedure A, 1. Inoculate into 7 ml sterile YM broth and incubate 10 h. Immerse the sterile paper strips in this logarithmic-phase culture and, when they are uniformly wet, pipette excess liquid from tube. Transfer the paper strips aseptically to the sterile dessicant vial, cover with the layer of glass wool, and cap the tube lightly. Place the tube and contents in a protective container (desiccator) containing anhydrous $CaSO_4$ and allow to air dry slowly for 48 h, then store at 4° to 6°.

Nonpropagative maintenance of stock cultures on paper strips does not replace long-term preservation in the lyophilized state. As an alternative to propagative maintenance by serial subculture, however, it has distinct advantages. In contrast to the propagative technique, the paper-strip method avoids the opportunity for genetic change because culture degeneration is directly proportional to the number of generations through which the culture has been grown (*23*). For these two methods, the sequence of transfers involved in maintenance and development of inocula for production is compared in figure 2. The paper-strip technique provides reliable inocula of standardized genetic composition, size, and viability. It is also convenient and saves time, space, and equipment.

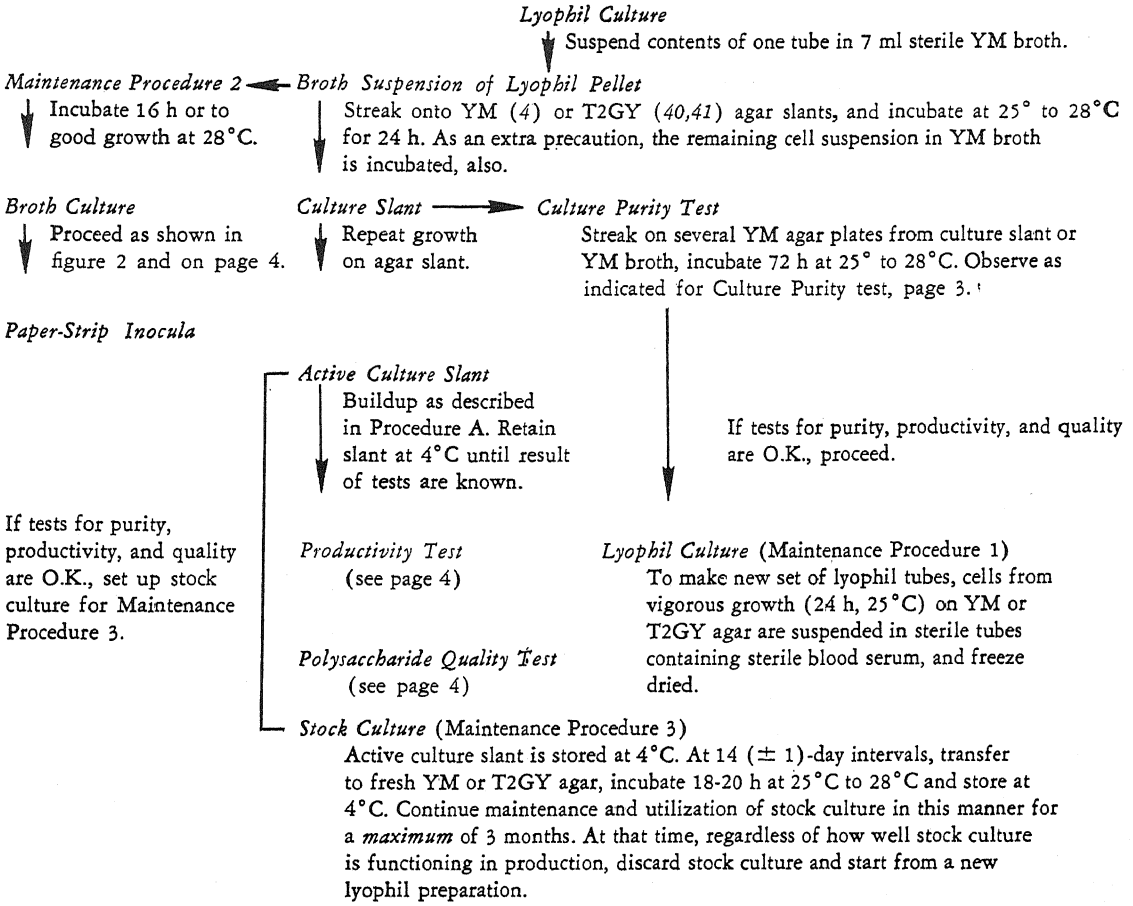
When maintained on paper strips, strain NRRL B-1459 is stable and viable for at least 3 months; however, after 3 months, the remaining paper strip inocula should be discarded and a new lot prepared.

Polysaccharide production from a paper strip inoculum (Procedure B) has been carried only through the Fernbach Productivity test stage, page 7. The results were satisfactory; continued observation should be made, however, of its use in production.

[4]Information through the courtesy of Dr. Denis K. Kidby, Soil Science and Plant Nutrition, University of Western Australia, Nedlands, W.A. Dr. Kidby conducted this research at NRRC as a foreign research associate, University of Guelph, Ontario, Canada.

[5]Dehydrating agent, Davison Chemicals, Baltimore, Md.

FIGURE 1.—Procedures for culture maintenance: Relations among three methods.



Procedure 3: Serial subculture on agar slants, semipropagative preservation

This procedure is detailed in figure 1 and compared with the technique of paper-strip preservation in figure 2. It is the method that has been used traditionally in microbial polysaccharide research at NRRC. When used with close adherence to prescribed details (*32,40*), it gave satisfactory results with strain NRRL B-1459 as evaluated by culture viscosity attained and yield (Productivity test, page 4 and page 7). Other culture-dependent criteria, established only recently as influencing solution properties of the xanthan product (*38*), were not considered. Because it is now known that semipropagative maintenance favors culture degeneration, this technique should be avoided. The procedure may serve, however, as an alternative for limited periods under circumstances where lyophilization equipment is not accessible.

Buildup of Inocula for Polysaccharide Production

Two general types of procedures are described: A and A′ (buildup starts from an agar slant) and B (buildup starts from a paper-strip inoculum). The general sequence of steps is shown in figure 2. Procedure A has been used primarily in the Engineering and Development Laboratory at NRRC (*40,41*), and procedure A′ has been used in the Fermentation Laboratory (*4,3*). The results obtained appear to be equivalent.

Procedure A: Inoculation from agar-slant culture

1. Inoculate (1 standard loopful, 2.5 to 3 mm diameter) from fresh (20 to 24 h old) YM (*4*) or T2GY (*40,41*) slant to 7-ml YM broth in 18- × 150-mm test tube(s). Incubate the tubes at 20° to 25° inclination, on either a reciprocal (about 100 strokes/min; about 6 cm amplitude) or rotary (about 160 r/min; 5 to 6.5 cm eccentricity) shaker for 24 h at 28° C.

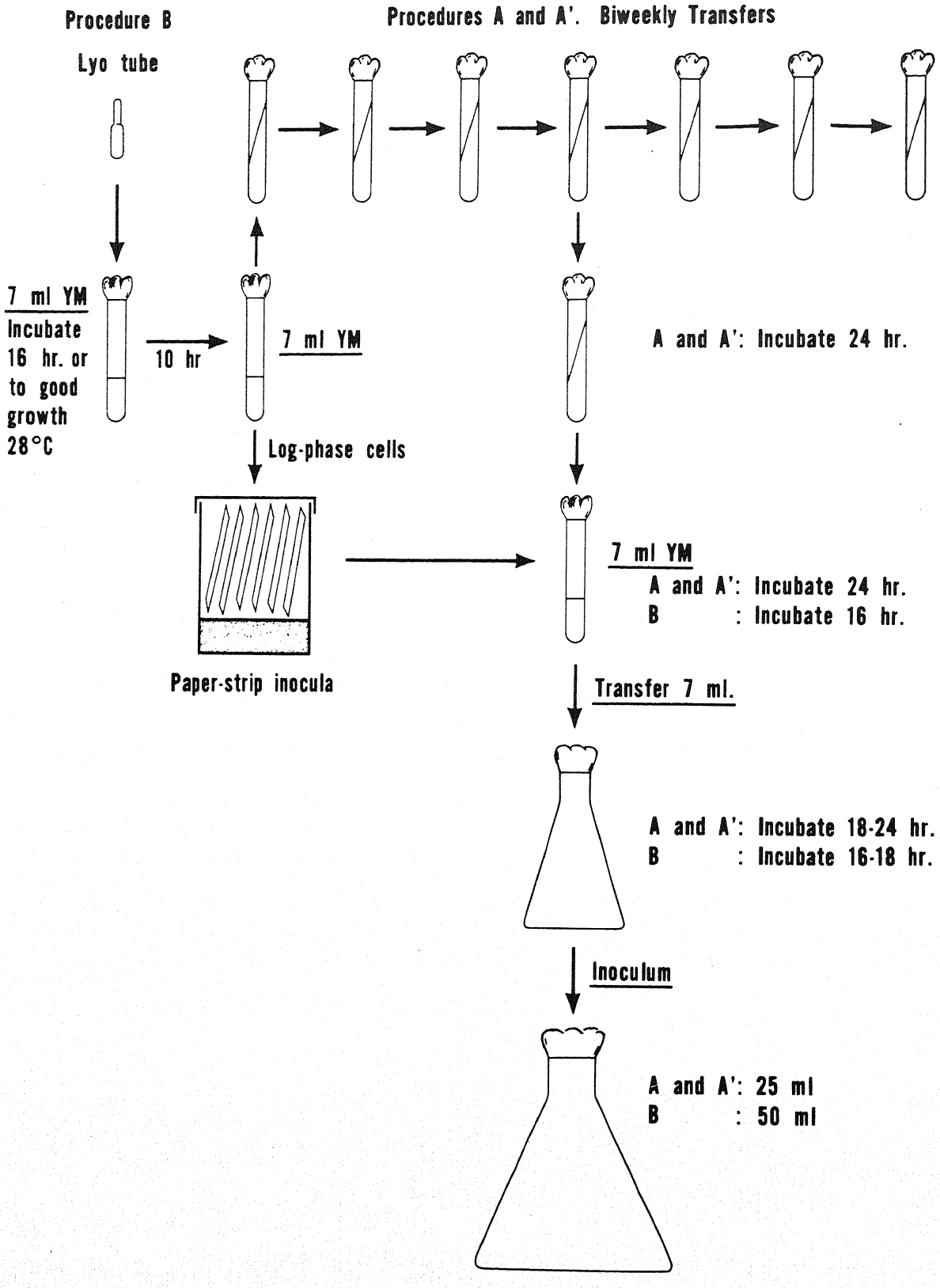


FIGURE 2.—Development of inocula for xanthan production:
Sequence of transfers starting with paper-strip or agar-slant stock cultures.

2. Transfer (7 ml) to 43-ml YM broth in 300-ml Erlenmeyer flask. Incubate 18 to 24 h on rotary shaker (200 to 245 r/min, 5.08 cm eccentricity) at 28° C. The pH of this culture is in the range 7.5 to 7.8.

This culture may be used to (a) inoculate culture medium for production or (b) to build up increased volume of inoculum as follows:

(a) Use culture 2 above in the proportion of 25 ml to 475 ml production medium. Percentage of inoculum is 5 percent and standard for the production stage. For laboratory-scale production, use a 2,800-ml Fernbach flask. The closure consists of two, 6-inch, single gauze-faced, fiber-bonded Rapid-Flo Filter Disks. Incubate 48 h on rotary shaker (200 r/min) at 28° C.

Production of this culture in Fernbach flasks and measurement of its viscosity, which should be at least 6,800 cPs, constitutes the Productivity Test. Conditions for viscosity measurement are stated under Fermentation Conditions. Usually under the conditions of this Productivity Test, viscosity of 6,800 to 7,000 cPs correlates with a yield of 1.3 to 1.5 g of xanthan per 100 g of fermentate. Yield analysis of xanthan is described under Analytical Methods, page 10.

(b) To build up increased volume of inoculum, transfer 25 ml of culture 2 above to 475 ml YM broth in a 2,800-ml Fernbach flask and incubate 24 h on rotary shaker at 28° C. For larger volumes of inoculum use multiple Fernbach flasks or small fermentor vessels.

For each production run, make a control Productivity Test on the inoculum.

Procedure A′: Inoculation from agar-slant culture

This alternate procedure for inoculum buildup is used in 10-l fermentations (*3*) or in flask fermentations using smaller volumes:

Stage 1 The cells contained on one fresh YM agar slant 20 to 24 h old are suspended in 7 ml of YM broth. Add 1 ml to 50 ml of YM broth in a 300-ml Erlenmeyer flask and incubate on a rotary shaker (175 to 200 r/min, 5.08 cm eccentricity) for 24 h at 28° C.

Stage 2 Transfer the 50 ml to 500 ml of YM broth in a 2,800-ml Fernbach flask and incubate 18 to 24 h on the rotary shaker, 28° C.

Stage 3 To inoculate 10-l of production medium contained in a 20-l fermentor at a rate of 5 percent v/v, add 500 ml (contents of flask) from stage 2 and incubate 48 h at 28° C. This type of vessel, the design and accessories of which have been described (*10*), requires baffles, agitation (150-200 r/min), and air (0.5-0.75 vol./l/min) supply. Agitation is then increased as the culture broth begins to thicken.

Procedure B: Inoculation from dry paper strip

1. Introduce one sterile inoculum strip [equivalent to about 1 standard loopful (2.5 to 3.0 mm diameter of broth culture)] into 7 ml YM broth, in a 18- × 150-mm test tube. Handle strip with forceps that have been dipped into ethanol and then flamed to remove adhering ethanol. Hold the tube containing broth and strip on a Vortex mixer for about 20 s and then incubate 16 to 18 h as stated for Procedure A, 1.
2. Transfer (7 ml) to 43 ml YM broth in a 300-ml Erlenmeyer flask. Incubate 24 h under condition Procedure A, 2.
3. Transfer (50 ml) to 500-ml medium in a 2,800-ml Fernbach flask and proceed as stated in Procedure A.

Fermentation Conditions and Scale of Operation for Batch Cultures

All fermentations for xanthan production are at 28° C for 48 h under conditions of agitation and aeration suitable for the specific equipment used. [Information is available on the measurement of oxygen absorption rate (*6,30,36*) and on the relation between vessel design and other factors on this rate (*6*).] The most desirable pH for xanthan production is 7.0 ± 0.5 and, although the biosynthesis progresses at somewhat lower pH, 6.0 to 6.2 is the lower limit (*32*).

At 48 h fermentation time, analyses should show

Viscosity: In the range of 6,800-7,800 cP (Brookfield viscometer LVT, 30 r/min, spindle 4, 25° C). Before measuring viscosity of the Fernbach-culture test for productivity, page 4, water is added to replace that lost by evaporation during incubation.

pH: Not lower than 6.0 to 6.2.

Reducing sugar: None.

Laboratory Scale

Our unit container is a 2,800-ml Fernbach flask that contains 10 g glucose in a total initial volume of 500 ml. The yield of dry purified product is about 6.0 to 6.5 g.

SemiPilot-Plant Scale

The design, accessories, and operation of 8-l glass fermentors (*40,41*) and 20-l stainless-steel fermentors (*10*), have been published. These are useful for experimentation (*32,40,41*) and production such as that described in Section V, Procedure A′ (*3*).

Pilot-Plant Scale

Operations in 60- and 600-gal fermentors have been described (*37,36*) and reviewed (*16,47*), including information on agitation, aeration, and oxygen absorption rate (*37*).

Isolating and Purifying Xanthan from Culture Fluid

Laboratory Scale

The procedure outlined results in a product of high purity suitable for most chemical and physical characterization. Products of lesser purity can be obtained by reducing the degree of dilution of the fermentate, number or gravitational force of centrifugations, number of reprecipitations, or the extent of dialysis or its omission. The nonsolvent indicated is ethanol; methanol would be equivalent. Other nonsolvents as isopropanol, isobutanol or acetone, remove less color and inorganic salt.

Quaternary ammonium halides may be used as precipitants (*1*); the amount retained in products purified to different extents has been measured and reported (*43*). Lyophilization is the only satisfactory method for dehydrating salt-free solutions of xanthan. In the absence of an electrolyte, xanthan cannot be precipitated from aqueous solution nor dehydrated by alcohol; in its presence, dehydration can be accomplished by either methanol or ethanol to yield a product of low salt content (*16,37,47*). The procedure would serve also to isolate purified xanthan from commercial products.

Semimicro Scale

Operations on this scale facilitate relatively rapid isolation and purification of xanthan samples from culture broths for the Polysaccharide Quality Test (page 4) and for pyruvate analysis (page 10) and gravimetric yield determination (page 10). Several procedures suggested here use the same principles as the Laboratory Scale but differ mainly in whether clarifica-

Step	*Operation*	*Remarks*
1	Dilute culture to <100 cP with 33 percent ethanol	Dilute 35 ml 95 percent ethanol to 100 ml
2	Centrifuge to remove cells and suspended matter from medium	For volumes of about 2 l or more, initial clarification is achieved with a continuous-flow Sharples supercentrifuge [standard laboratory-size unit, 2-in bowl, operated at 50,000 r/min (62,000 × G)] and a feed-rate of 100 to 150 ml/min. For smaller volumes, use a Spinco preparative ultracentrifuge (achieving about 40,000 × G) for 30 min. Instead of centrifugation, graded micropore filtration is feasible for small volumes. The diluted culture is passed through a prefilter, which removes most of the suspended matter, then through an 8.0 μ (and, if desired, 1.2 or 0.8 μ) filter.
3	Add KCl to make 1 percent concentration based on water volume	
4	Precipitate by addition of 95 percent ethanol	Requires 1 volume of additional ethanol per volume water [total ethanol concentration required is 55 percent (v/v) expressed as "absolute"]
5	Collect precipitate by Sharples or other centrifugation	
6	Break up gummy mass and slurry in 70 percent ethanol	This allows large masses to be separated without hydrating. Allow time for masses to soften so their size can be decreased by stirring. Good dispersion at this stage greatly facilitates the next step
7	Redissolve, dilute to <50 cP	Use 33 percent ethanol again, add slowly with vigorous stirring. Time is required (as refrigerating overnight) for particles to hydrate uniformly. Hydration and dispersion of particles is easier when solution is dense rather than thin. Intermittent mechanical stirring is beneficial.
8	Recentrifuge if Sharples had been used in step 2	One time at about 150 ml/min or two times at 300 ml/min
9	Reprecipitate, collect in Sharples or other centrifuge	As before, with 1 percent KCl and 55 percent ethanol
10	Slurry, redissolve; dilute to ⅓ of former volume	As before
11	Reprecipitate	As before
12	Redissolve, dilute to 300-500 cP	About 0.25 percent conc. at this viscosity
13	Dialyze, deionized water [Visking (Union Carbide)] 1-⅞ S.S. Dialysis Tubing is satisfactory. Soak to remove soluble matter before use	About 5 volumes water per volume of retentate; continue until dialyzate has resistance of 200,000 to 300,000 ohms after 24 h.
14	Adjust pH of retentate to 6.5	Use dilute KOH; pH before adjustment will be 4 to 4.5 if dialysis was complete
15	Filter with minimum suction through coarse sintered glass funnel	Remove lint and other extraneous particles, and small amount of proteinaceous material that flocculates during step 13.
16	Concentrate to about 1 percent solution	
17	Lyophilize	About 300 ml solution per 1-l flask
18	If optical clarity is required, samples of this material at ca 0.1 percent concentration should be ultracentrifuged or ultrafiltered as needed.	

tion is by centrifugation or filtration. Attention is given to minimizing mechanical losses that are inherent in working with small samples of viscous polysaccharides.

For convenience, start with 10 g of fermented broth and dilute to 30 g with distilled water. Usually from a fermentation broth having 6,800 to 7,000 cP viscosity, our yield of xanthan, exclusive of mechanical losses, would be about 130 to 150 mg.

Clarification by high speed centrifugation is accomplished by scaling down the Laboratory procedure. Decant supernatant into centrifuge tube or bottle. Subsequent steps of precipitation and two reprecipitations of the polysaccharide as well as deionization by slurry with exchange resin may be conducted in this container. Alternatively, the solution could be freed of salt by dialysis and then lyophilized.

When clarification is to be done by filtration, 10 g of fermented broth is weighed into a beaker and diluted to 30 g with distilled water. The dilute solution is passed serially (aided by suction) through a cellulose prefilter, 1.8- or 0.8-, or both, and 0.65 μ Millipore filters of 47 mm diameter. Gel that accumulates on the filter is mixed with drops of water until it passes through, or it is transferred to a filter of larger pore size. Rinsing the beaker and filters with water increases the weight of the filtrate to about 50 g.

This solution is transferred to the beaker. The flask is rinsed thrice with 95 percent ethanol (to make polysaccharide adhering to wall insoluble, visible, and loosened from walls) and once with water. The rinsings are added to the beaker. On the basis of estimated water volume, the contents of the beaker are adjusted to 1 percent KCl by adding saturated KCl solution and about 65 percent ethanol by adding 2 vol. of 95 percent ethanol.

The precipitated polysaccharide is collected with a stirring rod and 20-mesh stainless-steel screen, redissolved in 50 ml deionized water, and reprecipitated. Reprecipitation is repeated from a smaller volume of water and lower salt concentration. The precipitate is slurried and soaked 10 to 20 min in 80 percent ethanol to leach out salt, pressed free of excess solvent in a 1-dram vial, and teased apart. The sample and vial are dried by holding *in vacuo* at room temperature in the presence of P_2O_5 for 0.5 h or longer and then heated at 100° for 1 h (see Analytical Methods, moisture content).

Mechanical losses by the filtration procedure may be 10 to 15 percent and by the centrifugation technique, somewhat less. As previously reported (*30,37*), gravimetric determination of the xanthan content of culture liquors is rapid with little loss. The product, however, is not pure enough to be used for pyruvate analysis.

Pilot-Plant Scale

Procedures have been reported for isolation and purification of xanthan by use of methanol (*37*) or quaternary ammonium compounds (*1*) and dehydration by methanol (*37*). The whole fermented broth may be spray- or drum-dried (*36,47*). Reviews and cost comparisons also have been published (*16,47*).

Hygroscopicity and Dispersibility of Xanthan

Hygroscopicity—Xanthan, as either powder or fine fibrous particles, is hygroscopic. The moisture content is proportional to the relative humidity (R.H.) to which it has been exposed and, at about 50 percent R.H. and 20° C, may be 13 to 15 percent. Two alternatives accurately determine dry sample weight:

1. Humidify and store the stock xanthan sample in a room at constant temperature and humidity (50 percent R.H., 20° C) make all weighings in that room, analyze a separate sample for moisture content (page 10), and calculate all other sample weights to dry basis. This procedure permits research on xanthan that has not been subjected to heat. However these moisture-containing samples undergo spontaneous deacetylation and possibly other hydrolytic changes during long storage.
2. Dry the sample for experimentation as stated in Analytical Methods. More than 95 percent of the moisture is removed under high vacuum; the sample is subjected to heat only to remove last traces of moisture.

Some observations on the effect of heating moisture-containing xanthan solid have been reported (*7,49*). No specific information is available on the influence of commercial drying procedures on xanthan properties.

Dispersibility—People inexperienced in dispersing polysaccharide hydrocolloids complain about problems encountered in dispersing commercial xanthan. However, we have been unable to alter commercial preparations in any way to prevent their dispersing quite readily. The problem appears related to the technique of dispersion more than to the product. Although techniques described here are primarily for the laboratory, the basic principles are generally applicable. Especially important is to combine xanthan and water so as to prevent clumping and to recognize that hydration and dispersal are easier when the solution is dense rather than thin.

An expansion of the initially stated technique (*22*) follows:

The method used depends on the volume of the final solution. If a small solution is to be prepared in a vol-

umetric flask, for example, slow addition of water accompanied by vigorous shaking will give the best results. However, adding a small amount of water and allowing the mixture to stand overnight before diluting works best of all. If the final volume is large enough to prepare in a beaker with mechanical stirring, cautious addition of the dry powder to the water, with stirring, is more convenient and requires less time. Addition of the powder must be gradual enough to avoid clumping, which will impede hydration. As the particles hydrate and swell, they become nearly transparent, so observe the drainage of the solution on the side of the container to determine whether dispersion is complete.

Solubilization can also be enhanced by heating, although heating results in changes in physical properties that are not well understood (*14,22,34*). Two satisfactory techniques are:

1) A dispersion, prepared as described above, may be heated to about 85° C in a boiling water bath or by steam and then cooled quickly (*22*).

2) Dry powder may be stirred slowly into water at about 60° to 70° C. After the polysaccharide addition is complete, the solution is allowed to cool spontaneously.

A propellor-type agitator (Cowles Dissolver) is used to disperse xanthan on the pilot-plant scale.

Analytical Methods

Residual Glucose

Glucose in culture liquor may be estimated with Tes-Tape (urine sugar analysis paper), which is specific for glucose. More precise determination can be made by any standard reducing sugar method (*30,37*) or by enzymic analysis (glucose oxidase). Commercial kits for enzymic glucose determination, such as Glucostat (Worthington Biochemical Corporation, Freehold, N.J.), are available with complete instructions.

Xanthan Content

In culture liquors several methods are available for determining total polysaccharide present in the whole liquor. The metachromatic method of Egle and Finn as described by Moraine and Rogovin (*32*) is specific for anionic polysaccharide. Gravimetric determination of partially purified polysaccharide may also be used (*30,37*). If residual glucose is being determined on the same sample, the phenol-sulfuric acid spectrophotometric analysis (*8*) for total carbohydrate can be used. Pure xanthan is used as a standard for this analysis, and a correction is made for the amount of residual glucose found.

In solutions of pure xanthan determination can be made directly by the phenol-sulfuric acid method described above.

Xanthan Yield

The percentage yield of xanthan = [dry-weight of xanthan/weight of glucose ($C_6H_{12}O_6$) in production medium] × 100.

Typical yields of recovered dry xanthan are

Pilot-plant scale	60-70 percent
Laboratory scale, well purified	60 percent

Moisture Content

Duplicate samples (about 0.3 g) of xanthan powder are held either under high vacuum at room temperature for several hours or overnight in an Abderhalden drier in the presence of P_2O_5 or in a vacuum oven. The samples are then heated at 80° to 90° C for about 1 h before weighing.

Pyruvate Content

Pyruvic acid occurs in xanthan as the 4,6-*O*-(1-carboxyethylidene) derivative (*11,44*). It can be determined in a hydrolyzate of xanthan either by enzymic (*9*) or colorimetric (as the 2,4-dinitrophenylhydrazone derivative) procedures (*45*). At NRRC an enzymic method is used which is essentially the same as that already described (*9*). The materials required and the procedure are as follows:

Materials

Lactate dehydrogenase enzyme: We use a suspension of 36.5 mg enzyme per ml ammonium sulfate solution, obtained from Worthington Biochemical Corp. with an activity of 61 units per mg. Any comparable product from other suppliers is satisfactory; reaction time may vary depending upon the source from which the enzyme was isolated.

Nicotinamide adenine dinucleotide, reduced (NADH), 10 mg/ml in 0.1 percent sodium bicarbonate.

Triethanolamine, 1 *N* solution in water.
Hydrochloric Acid, 1 *N* solution.
Sodium Carbonate, 1 *M* solution
Purified Xanthan.

A 4 to 5 mg sample is required that contains xanthan of dry-weight known with an accuracy of about ± 5 percent. The sample must be essentially free of cells, other suspended matter, salts, and other soluble residual

from the culture broth. Xanthan products isolated in the regular way meet these requirements. When pyruvic acid determination is needed for the Polysaccharide Quality Test a special sample of adequate purity may be isolated quickly as described in Semimicro Scale, page 8.

Procedure

Dissolve 4 to 5 mg of xanthan (dry weight basis) in 2ml HCl. Cap tube tightly and heat at 100° C for 3 h. Neutralize acid with 2 ml Na_2CO_3 and dilute mixture to 10 ml. Pipette 2 ml of mixture into a spectrophotometer cell with a 1 cm path length, add 1 ml triethanolamine solution, and 50 μl NADH solution. Shake cell thoroughly. Measure absorbance at 340 nm (nanometer) against a blank containing no NADH. Add 4 μl enzyme suspension and shake mixture again. Measure absorbance at 5-min intervals until no further decrease in absorbance occurs, usually 20 to 25 min. Subtract final absorbance from initial absorbance to determine how much NADH has been consumed. Multiply the decrease in absorbance by 43.24 to obtain amount of pyruvic acid in μg. Percent pyruvic acid is obtained by multiplying μg pyruvic acid by 5 (because a 2-ml aliquot from a 10 ml total volume was used) and by dividing by μg xanthan hydrolyzed.

Analysis of Other Constituents

Methods requiring hydrolysis of polysaccharide

The standard technique for polysaccharide hydrolysis is to heat a 1 percent solution in 1 N HCl at 100° C for 3 h. After treatment with Ag_2CO_3 followed by H_2S, the hydrolyzate is free of both Cl^- and Ag^+.

Carbohydrate composition is determined in hydrolyzates by either of two methods:

1) Paper chromatographic separation of the components, elution of individual sugars from the chromatogram, and phenol-sulfuric acid analysis of the eluate (*42*).

2) If the polysaccharide hydrolyzate is reduced with ^3H-sodium borohydride prior to chromatography, sugars can be determined directly on the chromatogram by liquid scintillation counting (*24*).

Methods not requiring hydrolysis

1) Uronic acid content can be measured by the carbazole method (*26*).

2) *O*-Acetyl is determined by the hydroxamic acid method (*28*).

Analytical Values

Xanthan from normal, large-colony cultures and well-purified in the laboratory shows values as follows:

	Percent
Nitrogen	0.08-0.10
Phosphorus	.03
Ash (sulfated)	13.0
Glucuronic acid	17.5
Pyruvic acid	4.5
O-Acetyl	4.5

The theoretical sulfated ash for xanthan isolated as described here (neutral K salt) and having 17.5 to 17.6 percent glucuronic acid and 4.5 percent pyruvic acid is in the range 12.5 to 13.5 percent.

A correct percentage of D-glucuronic acid assures that the contents of D-glucose and D-mannose also are normal and need not be measured routinely. The molar ratio of the components of xanthan, D-glucose:D-mannose:D-glucuronic acid:*O*-acetyl:pyruvic acid, is 2:2:1:1:0.30-0.35 (*24,42*). The ratio of the hexose components appears to be constant; variability has been observed in the substituent *O*-acetyl and pyruvate (*4,38*). The content of pyruvate is abnormally low in the polysaccharide produced by certain variant strains of NRRL B-1459 (*4,38*).

Structure and Molecular Weight of Xanthan

Based on research at NRRC, a tentative structure was proposed (*46*) and found to agree with the results of methylation structural analysis (*39*). Further research by others (*15,29*), employing new and more definitive methods, has established that xanthan consists of a linear backbone of glucose residues linked as in cellulose with alternate residues having appended a three-unit long side chain (fig. 3).

Side chains are attached to the linear backbone through D-mannose residues that are substituted by *O*-acetyl at their carbon-6 position. The maximum *O*-acetyl content found thus far corresponds to 1 mole per each of these mannose residues (*25*). D-mannose residues also terminate side chains. Part of these terminal D-mannose residues are substituted by pyruvic acid and are present as 4,6-*O*-(1-carboxyethylidene)-D-mannose (*15,29*). In polysaccharide produced by normal large colonies, the maximum pyruvate content found corresponds to 1 mole per two side chains (*25*). In polysaccharide produced by small, mucoid colonies (*4*), the pyruvate content usually corresponds to 1 mole per four side chains (*25*).

The regularity of distribution of side chains and of pyruvate in the macromolecule has not yet been established experimentally.

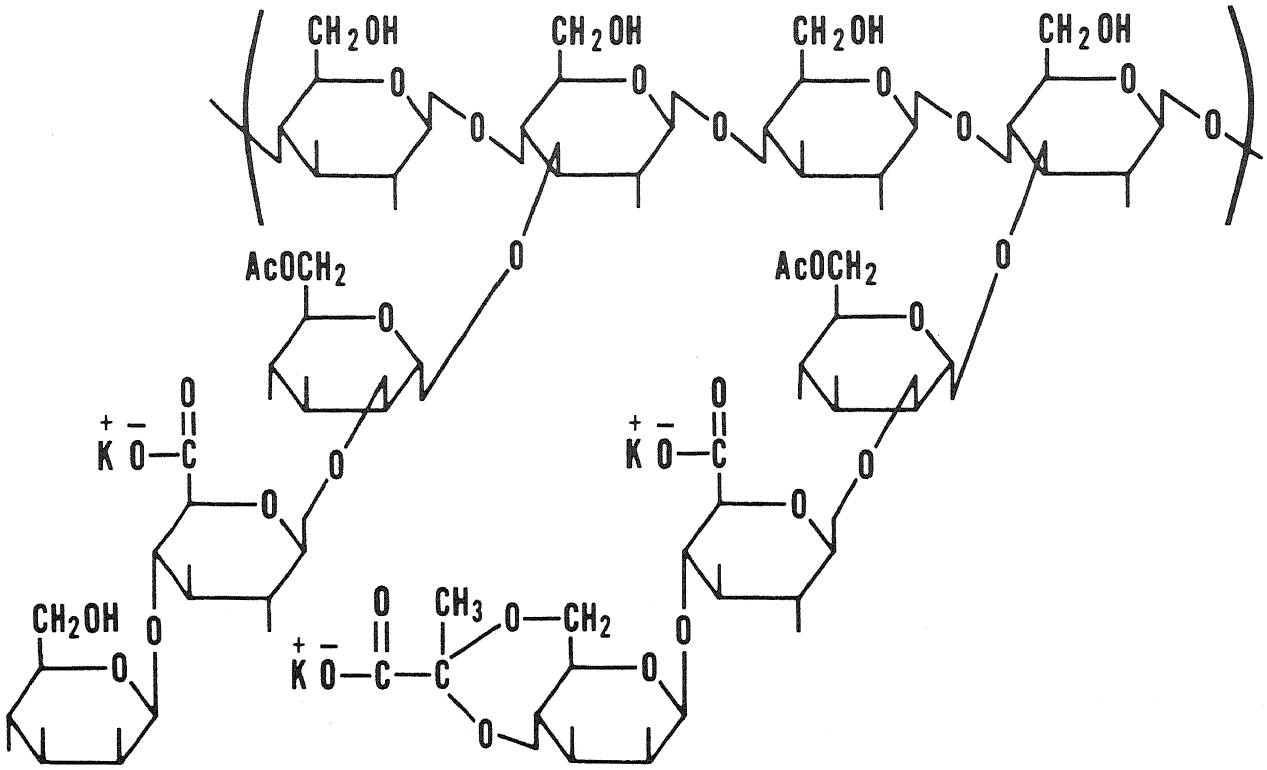


FIGURE 3.—A repeating unit of polysaccharide from strain NRRL B-1459 showing structural features known for the product of normal large colony cultures. Regularity of distribution of side chains and of pyruvate substituent have not been established experimentally.

Xanthan from *Xanthomonas campestris* NRRL B-1459 was the first extracellular microbial polysaccharide shown to have pyruvate as a substituent (*45*). Substituent pyruvate, however, is now established in extracellular polysaccharides from most *Xanthomonas* species (*33*), from many Rhizobiums, and from numerous other genera.

Xanthan has been estimated to have a molecular weight of about 5×10^6 by light scattering and sedimentation analysis (*7*). These NRRC investigators also reported polydispersity in their solutions. Another investigator found a narrow distribution of particle size using membrane partition chromatography (*14*).

Reviews on Properties and Uses of Xanthan and Other Microbial Polysaccharides of NRRC Research

All fundamental characteristics of xanthan and its solutions relate significantly to the design and operation of equipment for its fermentation, recovery, and in many cases for its applications. References (*17,18,19,20*) are included here, therefore, in which all the properties known are summarized and correlated with practical applications as indicated in patent and other literature. These reviews include information, also, on other extracellular microbial polysaccharides discovered in NRRC research.

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